



# *Cryptosporidium xiaoi* n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (*Ovis aries*)

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## ABSTRACT

A new species, *Cryptosporidium xiaoi*, is described from sheep. Oocysts of *C. xiaoi*, previously identified as the *Cryptosporidium bovis*-like genotype or as *C. bovis* from sheep in Spain, Tunisia, United Kingdom, and the United States are recorded as such in GenBank (EU408314–EU408317, EU327318–EU327320, EF362478, EF514234, DQ991389, and EF158461). Oocysts obtained from naturally infected sheep were infectious for a lamb and oocysts from that lamb were infectious for three other lambs. The prepatent period for *C. xiaoi* in these four *Cryptosporidium*-naïve lambs was 7–8 days and the patent period was 13–15 days. Oocysts are similar to those of *C. bovis* but slightly smaller, measuring 2.94–4.41  $\mu\text{m} \times 2.94$ –4.41  $\mu\text{m}$  (mean = 3.94  $\mu\text{m} \times 3.44 \mu\text{m}$ ) with a length/width shape index of 1.15 ( $n = 25$ ). Oocysts of *C. xiaoi* were not infectious for BALB/c mice, *Bos taurus* calves, or *Capra aegagrus hircus* kids. Fragments of the SSU-rDNA, HSP-70, and actin genes were amplified by PCR, purified, and PCR products were sequenced. The new species was distinct from all other *Cryptosporidium* species as demonstrated by multi-locus analysis of the 3 unlinked loci. Based on morphological, molecular and biological data, this geographically widespread parasite found in *Ovis aries* is recognized as a new species and is named *C. xiaoi*.

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## 1. Introduction

Ovine cryptosporidiosis, first described in diarrheic lambs in Australia (Baker and Carbonell, 1974), has subsequently been reported in 12 other countries (reviewed by Santín and Trout, 2008). The highest prevalence of infection has been found most often in lambs (Abd El-Wahed, 1999; Majewska et al., 2000; Sturdee et al., 2003; Santín et al., 2007), especially those less than 1 month of age (Causapé et al., 2002; Misic et al., 2006). Most studies of cryptosporidiosis in sheep that were conducted before molecular testing assumed that sheep were infected with only one species, *Cryptosporidium parvum*, but with the application of molecular testing the

following species and genotypes have been detected in sheep feces: *C. parvum*, *C. suis*, *C. andersoni*, *C. hominis*, *C. bovis*, *C. bovis*-like, cervine genotype, novel bovine B genotype, pig genotype II, marsupial genotype, novel sheep genotype, and a unique unknown genotype (Morgan et al., 1998; McLauchlin et al., 2000; Chalmers et al., 2002; Ryan et al., 2005; Navarro-i-Martinez et al., 2007; Santín et al., 2007; Soltane et al., 2007; Elwin and Chalmers, 2008; Geurden et al., 2008; Mueller-Doblies et al., 2008). The novel sheep genotype as well as the unidentified *Cryptosporidium* reported in sheep by Chalmers et al. (2002) were further investigated and were identified as *C. bovis*/*C. bovis*-like and as the cervine genotype (Elwin and Chalmers, 2008). The species *C. suis*, *C. andersoni*, and *C. hominis* were each found in the feces of only one or two sheep (Ryan et al., 2005) and might represent oocysts passing through the intestinal tract as opposed to actual infections. The pig genotype II and marsupial genotype

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that were each found in only four specimens might also represent oocysts simply passing through the intestinal tract (Ryan et al., 2005).

Oocysts identified as *C. bovis* or as the *C. bovis*-like genotype have been reported in sheep in Spain (Navarro-i-Martinez et al., 2007), Tunisia (Soltane et al., 2007), the United Kingdom (UK) (Elwin and Chalmers, 2008; Mueller-Doblies et al., 2008), and the United States (US) (Santín et al., 2007). A novel bovine B genotype (*C. bovis* was previously reported as the bovine B genotype) was reported in sheep in Australia (Ryan et al., 2005). The *C. bovis*-like genotype also was reported from a goat and a yak in China (Feng et al., 2007; Karanis et al., 2007). The *C. bovis*-like genotype and *C. bovis* are genetically very similar. Identification as the *C. bovis*-like genotype is based solely on the nucleotide sequence similarity of a fragment of the SSU-rDNA gene compared with *C. bovis* found in cattle and with the *C. bovis*-like genotype in other hosts. Oocysts of *C. bovis* isolated from cattle were not infectious for two experimentally exposed lambs less than 1 week of age and were not detected in 42 lambs 2–3 months of age, but were detected in a 2-week-old lamb (Fayer et al., 2005).

To determine if the *C. bovis*-like genotype was a slight variant of *C. bovis* or a distinct species, segments from two additional unlinked loci, the HSP-70 and actin genes, were sequenced and compared with those of *C. bovis*. Furthermore, as proposed by Xiao et al. (2004) for qualification of species status, morphometric and biological data were obtained. Data obtained in the present study indicate that the *C. bovis*-like isolate from sheep qualifies as a new species and as such is named *Cryptosporidium xiaoi* in honor of Dr. Lihua Xiao for his many contributions to the taxonomy and molecular epidemiology of *Cryptosporidium* species.

## 2. Materials and methods

### 2.1. Experimental design

Fecal specimens were collected from ewes in a commercial flock and from mixed age sheep in an experimental flock at the Agricultural Research Service, Beltsville Agricultural Research Center (BARC) to obtain the initial *C. xiaoi* oocysts for the present study. *C. xiaoi* oocysts from four naturally infected sheep were pooled and used to infect a single lamb (Lamb 1). Oocysts from that lamb were used to infect three additional lambs (Lambs 2–4). Oocysts from the three lambs were used to infect 2 calves, 3 goats, and 12 mice. Oocysts from these lambs were measured and photographed. Oocysts from every infected animal were observed by microscopy and confirmed by PCR followed by gene sequencing as described below.

### 2.2. Oocyst collection

To obtain oocysts for microscopic examination and for DNA extraction, feces were processed as described (Fayer et al., 2000). Fifty-three fecal specimens were collected from the ground of a farmyard pen of ewes in Union Bridge, Maryland, placed in specimen cups and transported to the laboratory for processing. Nineteen fecal specimens were collected from a pasture where mixed age sheep were held

at the BARC. Feces from experimentally exposed lambs, calves, and goats were collected daily for 21–24 days beginning on the day of inoculation and placed in individual specimen cups with lids, labeled with the date and animal number, and held at 4°C until processed. Five to 15 g of feces from ewes, lambs, calves, and goats were transferred from each specimen cup to a 50 ml centrifuge tube containing approximately 35 ml distilled water (dH<sub>2</sub>O). The tube was capped and contents were thoroughly mixed using a Vortex-Genie (Scientific Industries, Bohemia, New York). To remove large particles the fecal suspension was sieved through a 45 µm pore size wire screen. The sieved suspension was placed in another 50 ml tube and the final volume was adjusted to 50 ml with dH<sub>2</sub>O. The tube was centrifuged at 1800 × g for 15 min, supernatant was discarded, and the pellet, suspended in 25 ml dH<sub>2</sub>O, was mixed by Vortex-Genie. Twenty-five millilitres of CsCl (1.4 g/l) was added to the tube, the contents were mixed thoroughly, and the tube was centrifuged at 300 × g for 20 min. Four millilitres of supernatant were aspirated from the top of the tube and transferred to a 15 ml centrifuge tube where dH<sub>2</sub>O was added to reach a final volume of 15 ml. The tube was centrifuged at 1800 × g for 15 min and similarly washed twice with dH<sub>2</sub>O before the final pellet was suspended in 500 µl of dH<sub>2</sub>O. Portions of the suspension were examined by immunofluorescence microscopy (IFA), differential interference microscopy, and molecular methods as described below.

### 2.3. Microscopic examination of oocysts

A 2 µl aliquot of cleaned oocyst suspension was mixed with 2 µl of premixed anti-*Cryptosporidium* reagent (MerlFluor™, Meridian Biosciences Inc., Cincinnati, Ohio). Two microlitres of this mixture was pipetted into a well (11 mm diameter) of a 3-well glass microscope slide and the slide covered with a coverslip. The well areas were examined using a Zeiss Axioskop equipped with epifluorescence and an FITC-Texas Red™ dual wavelength filter. Twenty five oocysts were measured by ocular micrometer at 100×. For purposes of comparison, oocysts (*n* = 25) of *C. parvum* from a bovine source were measured using the same microscope. Using a Zeiss Axioskop microscope with differential interference contrast microscopy, photomicrographs of *C. xiaoi* oocysts were obtained and deposited as phototypes in the US National Parasite Collection, Beltsville, MD as accession no. USNPC 101171.

### 2.4. DNA extraction, PCR, and sequence analyses

DNA was extracted from each 50 µl suspension of cleaned oocysts using a DNeasy Tissue Kit (Qiagen, Valencia, California). To increase the amount of DNA that could be recovered, nucleic acid was eluted in 100 µl of elution buffer included in the DNeasy Kit.

PCR was used to amplify fragments of the SSU-rDNA (~830 bp), HSP-70 (~325 bp), and actin (~1066 bp) genes (Xiao et al., 2001). For the SSU-rDNA fragment, primary amplification employed the primers CryptoF: 5'TTC TAG AGC TAA TAC ATG CG3' and CryptoR: 5'CCC ATT TCC TTC GAA ACA GGA3'. Secondary amplification employed the

primers AL1598: 5'AAG GAG TAA GGA ACA ACC TCC A3' and AL3032: 5'GGA AGG GTT GTA TTT ATT AGA TAA AG3'. For the HSP-70 fragment, primary amplification employed the primers HSPF4: 5'GGT GGT GGT ACT TTT GAT GTA TC3' and HSPR4: 5' GCC TGA ACC TTT GGA ATA CG3'. Secondary amplification employed the primers HSPF3: 5'GCT GST GAT ACT CAC TTG GGT GG3' and HSPR3: 5'CTC TTG TCC ATA CCA GCA TCC3'. For the actin fragment, primary amplification employed the primers Actin1F: 5'ATC RGW GAA GAA GWA RYW CAA GC3' and Actin1R: 5'AGA ARC AYT TTC TGT GKA CAA T3'. Secondary amplification employed the primers Actin2F: 5'CAA GCW TTR GTT GTT GAY AA3' and Actin2R: 5'TTT CTG TGK ACA ATW SWT GG3'. For the SSU-rDNA gene primary PCR step, PCR mixture contained 1× PCR buffer, 3 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 2.5 U Taq, 2.5 µl of BSA (0.1 g/10 ml), and 1 µM for each forward and reverse primer in a total of 50 µl reaction volume. A total of 35 cycles, each consisting of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 1 min, were performed; an initial hot start at 94 °C for 3 min and a final extension step at 72 °C for 7 min were also included. For the secondary PCR step, the PCR mixture was identical except that a concentration of 1.5 mM MgCl<sub>2</sub> was used. A total of 40 cycles, each consisting of 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 2 min, were performed; an initial hot start at 94 °C for 3 min and a final extension step at 72 °C for 7 min were also included. For the actin gene primary PCR step, PCR mixture contained 1× PCR buffer, 3 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 2.5 U Taq, 2.5 µl of BSA (0.1 g/10 ml), and 1 µM for each forward and reverse primer in a total of 50 µl reaction volume. A total of 35 cycles, each consisting of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min, were performed; an initial hot start at 94 °C for 5 min and a final extension step at 72 °C for 10 min were also included. For the secondary PCR step, the PCR mixture was identical. A total of 35 cycles, each consisting of 94 °C for 45 s, 45 °C for 45 s, and 72 °C for 1 min, were performed; an initial hot start at 94 °C for 5 min and a final extension step at 72 °C for 10 min were also included. For the HSP gene primary PCR step, PCR mixture contained 1× PCR buffer, 1.75 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 2 U Taq, 2.5 µl of BSA (0.1 g/10 ml), and 0.5 µM for each forward and reverse primer in a total of 50 µl reaction volume. A total of 40 cycles, each consisting of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, were performed; an initial hot start at 94 °C for 5 min and a final extension step at 72 °C for 10 min were also included. For the secondary PCR step, the PCR mixture and conditions were identical.

PCR products were analyzed on 1% agarose gel, visualized by ethidium bromide staining, and purified with Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT™) (USB Corporation, Cleveland, OH). Purified products were sequenced in both directions. The same PCR primers were used in 10 µl reactions, Big Dye™ chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, CA).

## 2.5. Cloning

Nested PCR products from two isolates of *C. xiaoi* SSU-rDNA gene were cloned to ensure that one copy of this gene

was analyzed at a time to look for intra-isolate variations. The PCR products of SSU-rDNA from the 2 *C. xiaoi* isolates were cloned using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) and 8 clones for each of the isolates were sequenced using M13 forward and reverse primers in both directions.

## 2.6. Phylogenetic analyses

The SSU-rDNA, HSP-70, and actin sequences were compared with sequences from other *Cryptosporidium* species and genotypes from GenBank. *Plasmodium falciparum* was used as an out-group for HSP-70 (GenBank accession no. M19753), actin (GenBank accession no. M19146) and SSU-rDNA (GenBank accession no. M19172) analyses as in previous studies (Fayer et al., 2005, 2008; Ng et al., 2006). Sequences were aligned using the Clustal W algorithm in the Megalign module (DNASTAR Inc., Madison, WI). Clustal W determines that when a gap is inserted it can be removed only by editing, so final alignment adjustments were made manually to remove artificial gaps. Phylogenetic and molecular evolutionary analyses were made using MEGA version 3.1 (Kumar et al., 2004). Phylogenetic inference was by the Neighbor-Joining (NJ) method of Saitou and Nei (1987). Genetic distance was calculated with the Kimura 2-parameter model.

Nucleotide sequences of the SSU-rDNA, HSP-70, and actin genes of *C. xiaoi* have been deposited in GenBank under accession nos. FJ896041–FJ896053.

## 2.7. Infectivity for lambs, calves, goats, and mice

To prevent accidental infection with *Cryptosporidium* species in the environment, lambs, calves, and goats were housed on wood shavings in 3 m<sup>2</sup> pens with cement walls and floor in isolated cinderblock buildings. Animal caretakers wore new disposable coveralls, shoe covers, and gloves every time they entered the buildings. Water and hay were always available ad libitum.

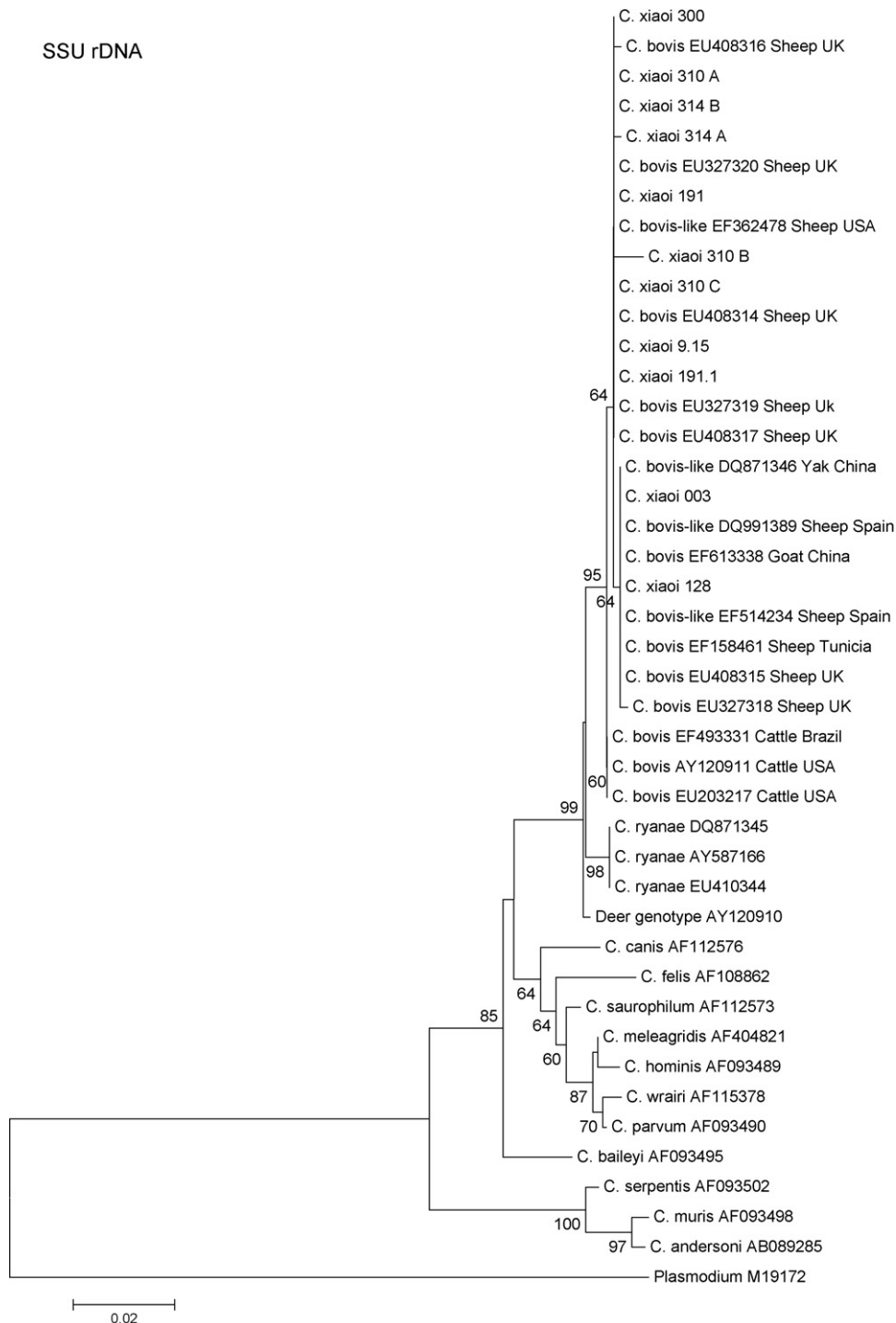
All lambs, calves, and goats were fed *C. xiaoi* oocysts suspended in water via a nipples bottle or syringe. Approximately 2000 oocysts of *C. xiaoi*, pooled from two fecal specimens obtained from a commercial sheep flock in Union Bridge, Maryland and from two additional specimens from an experimental flock at the BARC, were used to infect a 2-month-old male Suffolk lamb that had been housed in confinement, tested periodically, and found to be free of prior infection with *Cryptosporidium*. Feces were collected from that lamb (Lamb 1) for 23 consecutive days beginning on the day of infection.

*C. xiaoi* oocysts from Lamb 1 were pooled and used to infect three male Suffolk lambs from the BARC experimental flock. These lambs (Lambs 2–4) were held in individual pens in isolation buildings with their ewes until weaned and were found free of prior infection with *Cryptosporidium*. At 50–54 days of age each lamb received 4000–4500 *C. xiaoi* oocysts. Feces were collected from each lamb for the following 24 consecutive days.

Two male Holstein–Friesian calves taken from their dams at birth were fed calf milk-replacer twice daily. Calf 1 had been experimentally infected with *C. parvum*, had cleared

the infection, and was 83 days of age when fed 500,000 *C. xiaoi* oocysts. Calf 2 was 5 days of age when fed 100,000 *C. xiaoi* oocysts. Feces were collected from each calf daily for 23 consecutive days beginning the day oocysts were fed.

Three 36-week-old male Boer goat kids from the BARC herd were placed in individual pens in isolated buildings. Feces were collected daily for 1 week before they each received 10,000 *C. xiaoi* oocysts. Feces were collected from



**Fig. 1.** Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by a Neighbor-Joining analysis of a fragment of the 18S rRNA gene sequence, based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percent bootstrapping values from 1000 replicates.

each kid daily for 22 consecutive days beginning the day oocysts were fed.

Two litres of eight 5-day-old BALB/c mice, each with their dam, were purchased from the National Cancer Institute (Frederick, Maryland). Ten thousand oocysts of *C. xiaoi* were administered to each of six mouse pups per litre at 7 days of age by gastric intubation using a 26 ga gavage needle fitted to a micropipette. Two other mice each received 10,000 oocysts of *C. parvum* (Beltsville strain), obtained from experimentally infected calves, and served as positive controls, one mouse received no oocysts and served as a negative control. Mice were killed by CO<sub>2</sub> asphyxiation 5 days after intubation. The following tissues were taken from each mouse and subjected to DNA extraction and PCR as described above: stomach, duodenum, jejunum, and ileum.

### 2.8. Animal care

All housing, feeding, and experimental procedures involving lambs, calves, goats, and mice were conducted under protocols approved by the Beltsville Area Animal Care and Use Committee.

## 3. Results

### 3.1. Prevalence of *C. xiaoi* in flocks

Of 19 fecal specimens collected from sheep in an experimental flock at the BARC, six contained oocysts. Three contained oocysts of *C. xiaoi* and three contained *C. parvum*. Of 53 fecal specimens collected from ewes in a commercial flock in western Maryland 11 contained oocysts. Two specimens contained oocysts of *C. xiaoi* and nine contained *C. parvum*. All specimens containing oocysts of *C. xiaoi* had very few oocysts. After four of the five positive specimens were pooled approximately 2000 oocysts were recovered. All feces were firm and normal in appearance.

### 3.2. Transmission studies

Lamb 1 that received pooled *C. xiaoi* oocysts from the commercial and experimental flocks began excreting oocysts 8 days later and excreted oocysts for a period of 13 days. Collection of feces from small lambs is difficult and this lamb received a relatively small infectious dose, consequently only a few grams of feces were collected per day, amounting to approximately 15,000 oocysts from this lamb. Lambs 2, 3, and 4 began excreting *C. xiaoi* oocysts 7 and 8 days after exposure and continued excreting oocysts for 14 and 15 days. These lambs, infected with a larger number of oocysts, produced from 100,000 to >500,000 oocysts per lamb.

Oocysts were not detected in feces from the two calves or three goats infected with *C. xiaoi* oocysts by immunofluorescence microscopy or molecular methods.

Of the 12 mice intubated with 10,000 oocysts of *C. xiaoi* and killed 5 days later, DNA of *C. xiaoi* was not detected in any of their gastrointestinal tissues whereas DNA of *C. parvum* was detected in all intestinal tissues of the positive control mice. Tissues from the mouse that served as a negative control were all negative.

### 3.3. Oocyst characteristics

Oocysts of *C. xiaoi* were stained with anti-*Cryptosporidium* reagent and examined by fluorescence microscopy. They appeared pale in comparison with those of *C. parvum* which appeared bright green. Oocysts ( $n = 25$ ) of *C. xiaoi* measured  $2.94\text{--}4.41\ \mu\text{m} \times 2.94\text{--}4.41\ \mu\text{m}$  (mean =  $3.94\ \mu\text{m} \times 3.44\ \mu\text{m}$ ) with a length/width shape index of 1.15. Oocysts ( $n = 25$ ) of *C. parvum* measured  $3.68\text{--}5.15\ \mu\text{m} \times 3.68\text{--}4.41\ \mu\text{m}$  (mean =  $4.56\ \mu\text{m} \times 4.32\ \mu\text{m}$ ) and a length/width shape index of 1.06. Four sporozoites of *C. xiaoi* could be seen in a few oocysts by Nomarski interference contrast microscopy but because of the small oocyst size and indistinct internal contents sporozoites could not be clearly seen in most oocysts.

### 3.4. Gene sequence data

At the HSP-70 and actin genes, all *C. xiaoi* isolates examined were 100% identical. At the SSU-rDNA gene sequence variation among the *C. xiaoi* isolates was observed with a pairwise distance from 0 to 0.007. The sequences obtained from the clones of the SSU-rDNA gene from two isolates showed two and three different sequences for the two isolates examined (314: 314a and 314b; 310: 310a, 310b and 310c) (Fig. 1).

Partial sequences of the SSU-rDNA, actin, and HSP-70 genes were compared with *Cryptosporidium* sequence data obtained from GenBank. A Neighbour-Joining phylogenetic tree was constructed for the SSU-rDNA, actin, and HSP-70 genes. The phylogenetic relationships for these 3 genes were consistent with *C. xiaoi* forming a distinct cluster with *C. bovis*, *C. ryanae*, and the deer genotype with a bootstrap value of 99% for SSU-rDNA and HSP genes, and 100% for the actin gene (Figs. 1–3).

### 3.5. Description

#### 3.5.1. *Cryptosporidium xiaoi* n. sp.

Diagnosis	Oocysts are shed in feces fully sporulated. Oocysts ( $n = 25$ ) measure $2.94\text{--}4.41\ \mu\text{m} \times 2.94\text{--}4.41\ \mu\text{m}$ with a mean size of $3.94\ \mu\text{m} \times 3.44\ \mu\text{m}$ and a length/width shape index of 1.15. Endogenous stages are unknown.
Type host	Sheep ( <i>Ovis aries</i> )
Other natural hosts	Yak and goat (Feng et al., 2007; Karanis et al., 2007).
Experimental transmission	Attempts to infect lambs were successful. Attempts to infect cattle, goats and mice were unsuccessful.
Prepatent period	7–8 days
Patent period	13–15 days
Type locality	Beltsville, MD, USA



Other localities	Sheep: Spain, Tunisia, United Kingdom; yak and goat: China
Material deposited	A phototype and description of oocysts are deposited in the United States National Parasite Collection, Beltsville, MD as accession no. USNPC 101171.
Etymology	This species is named <i>C. xiaoi</i> in honor of Dr. Lihua Xiao who has contributed greatly to taxonomy and molecular epidemiology of <i>Cryptosporidium</i> species.

#### 4. Discussion

Oocysts of *C. xiaoi*, previously recognized as the *C. bovis*-like genotype, were found to be morphologically similar to those of *C. bovis* found in cattle and to the *C. parvum* and *Cryptosporidium* cervine genotype found to infect sheep. Contents of the *C. xiaoi* oocysts often appeared indistinct and only occasionally four distinct sporozoites were seen within an oocyst. Twenty-five oocysts of *C. xiaoi* had a mean size of  $3.94 \mu\text{m} \times 3.44 \mu\text{m}$  with a length/width shape index of 1.15 whereas, for size comparison, 25 oocysts of *C. parvum* obtained from a calf, processed, and measured under the same conditions in the present study had a mean size of  $4.56 \mu\text{m} \times 4.32 \mu\text{m}$  and a shape index of 1.06. The mean size for oocysts of *C. bovis* was found to be  $4.89 \mu\text{m} \times 4.63 \mu\text{m}$  (Fayer et al., 2005) which is larger than that of *C. xiaoi*. Oocysts of *C. parvum* and *C. bovis* had

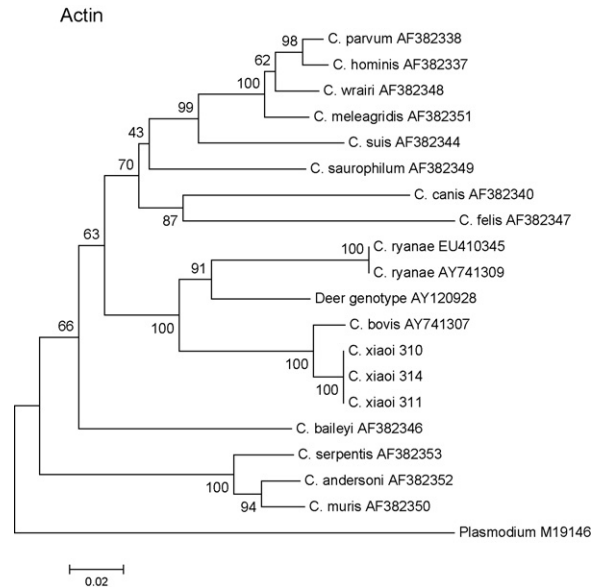


Fig. 2. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by a Neighbor-Joining analysis of a fragment of the actin gene sequence, based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percent bootstrapping values from 1000 replicates.

a shape index of 1.06 (Fayer et al., 2005). The color and thickness of the oocyst wall were indistinguishable among the 3 species however oocysts of *C. xiaoi* appeared much fainter than those of other species when viewed

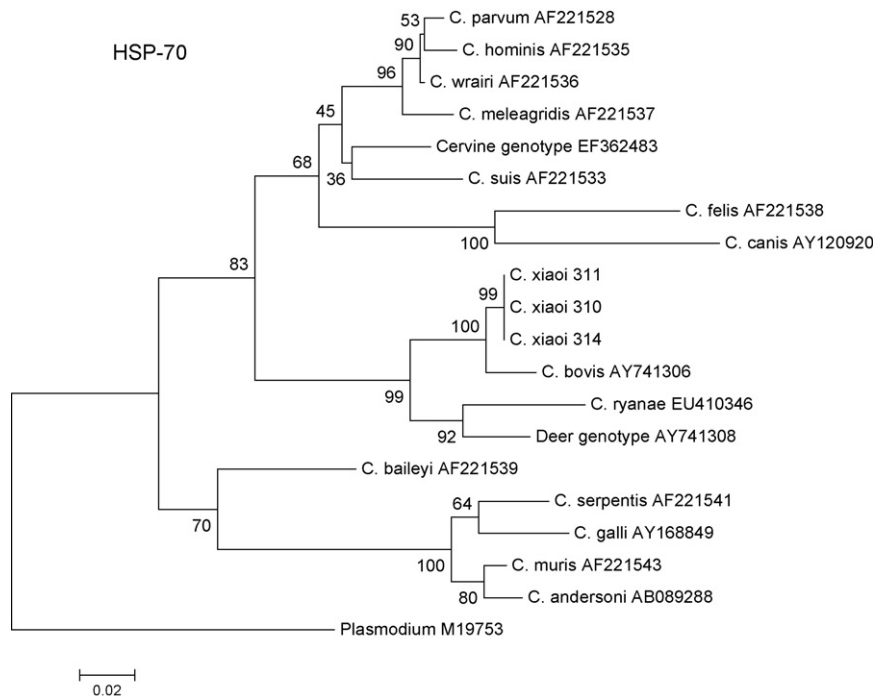


Fig. 3. Phylogenetic relationships among *Cryptosporidium* species and genotypes inferred by a Neighbor-Joining analysis of a fragment of the HSP-70 gene sequence, based on genetic distances calculated by the Kimura two-parameter model. Numbers on branches are percent bootstrapping values from 1000 replicates.

by fluorescence microscopy after staining with Merifluor antibody.

Genetically confirmed infections with *C. xiaoi*, previously referred to as the *C. bovis*-like genotype or *C. bovis* (in sheep), have been found in sheep the US, Spain, Tunisia, and the UK (Santín et al., 2007; Navarro-i-Martinez et al., 2007; Elwin and Chalmers, 2008; Mueller-Doblies et al., 2008) (Table 1). In the US, the presence of *C. bovis*-like was reported in 7 of 189 fecal specimens collected from asymptomatic sheep from a farm in Maryland (Santín et al., 2007). In Spain *C. bovis*-like oocysts were obtained from an 8-day-old lamb that died with massive diarrhea (Navarro-i-Martinez et al., 2007). In the UK, the *C. bovis*-like genotype was detected from 14 asymptomatic sheep in two different studies conducted in England, Wales, and Scotland (Elwin and Chalmers, 2008; Mueller-Doblies et al., 2008). The *C. bovis*-like genotype was reported from a yak (GenBank accession no. DQ871346) (Feng et al., 2007) and a goat (GenBank accession no. EF613338) (Karanis et al., 2007) in China. With only one animal of each species found infected confirmation and prevalence data from additional studies involving more animals will help to assess the relevance of these reports. A *Cryptosporidium* novel bovine B genotype reported in Australia in 14 sheep was probably *C. xiaoi*, however, we could not confirm the identity of this isolate because sequence data are not available for those samples in GenBank (Ryan et al., 2005).

Although the number of sheep previously reported to be naturally infected with *C. xiaoi* is quite small (4 in the US, 1 in Spain, 3 in Tunisia, and 14 in the UK), the age

distribution is great. Infected animals include lambs at 8, 14, and 21 days of age, and others at 2–48 months of age (Santín et al., 2007; Navarro-i-Martinez et al., 2007; Elwin and Chalmers, 2008; Mueller-Doblies et al., 2008). The only lamb infected with *C. xiaoi* that had signs of disease was the lamb in Spain but as the authors remarked “we have no proof that these symptoms were caused by infection with the *C. bovis*-like (*C. xiaoi*) organism.”

We observed sequence variability in the SSU-rDNA gene among the samples examined in our study (Fig. 1). Similar results were reported in the UK with four different sequences of the *C. bovis*-like genotype reported from the nine samples examined from sheep (Elwin and Chalmers, 2008). Also, in the UK, 2 different SSU-rDNA gene nucleotide sequences were reported in 5 infected sheep, in one of the lambs variation was observed in the nucleotide sequence over time (Mueller-Doblies et al., 2008). It is known that five copies of the SSU-rDNA gene are present in the *Cryptosporidium* genome, and previous studies have suggested that there is slight sequence heterogeneity in some of these copies (Le Blancq et al., 1997; Xiao et al., 1999). To ensure that one copy of the SSU-rDNA gene was analyzed at a time, single copies of the *C. xiaoi* SSU-rDNA gene were cloned into plasmid vectors from two isolates. We observed three and two different nucleotide sequences for each of the isolates cloned. Therefore, some of the sequence differences could be due to variation among different copies of rDNA gene.

In the present study phylogenetic analyses confirmed the validity of *C. xiaoi* at three independent loci. Datasets

**Table 1**

*Cryptosporidium bovis*, *C. bovis*-like, *C. ryanae*, deer genotype reported in GenBank from sheep, cattle, goat, deer, and yak using SSU-rDNA, actin, and HSP-70 genes.

Loci examined	Name	Host	GenBank	Country	Reference
SSU-rDNA	<i>C. bovis</i>	Sheep	EU408314	UK	Elwin and Chalmers (2008)
SSU-rDNA	<i>C. bovis</i>	Sheep	EU408315	UK	Elwin and Chalmers (2008)
SSU-rDNA	<i>C. bovis</i>	Sheep	EU408316	UK	Elwin and Chalmers (2008)
SSU-rDNA	<i>C. bovis</i>	Sheep	EU408317	UK	Elwin and Chalmers (2008)
SSU-rDNA	<i>C. bovis</i>	Sheep	EU327318	UK	Mueller-Doblies et al. (2008)
SSU-rDNA	<i>C. bovis</i>	Sheep	EU327319	UK	Mueller-Doblies et al. (2008)
SSU-rDNA	<i>C. bovis</i>	Sheep	EU327320	UK	Mueller-Doblies et al. (2008)
SSU-rDNA	<i>C. bovis</i> -like	Sheep	EF362478	USA	Santín et al. (2007)
SSU-rDNA	<i>C. bovis</i> -like	Sheep	EF514234	Spain	Navarro-i-Martinez et al. (2007)
SSU-rDNA	<i>C. bovis</i> -like	Sheep	DQ991389	Spain	Navarro-i-Martinez et al. (2007)
SSU-rDNA	<i>C. bovis</i>	Sheep	EF158461	Tunisia	Soltane et al. (unpublished)
SSU-rDNA	<i>C. bovis</i>	Goat	EF613338	China	Karanis et al. (2007)
SSU-rDNA	<i>C. bovis</i> -like	Yak	DQ871346	China	Feng et al. (2007)
SSU-rDNA	<i>C. bovis</i> (bovine B)	Cattle	AY120911	USA	Xiao et al. (2002)
SSU-rDNA	<i>C. bovis</i>	Cattle	AY741305	USA	Fayer et al. (2005)
SSU-rDNA	<i>C. bovis</i>	Cattle	EU203217	USA	Feltus et al. (2008)
SSU-rDNA	<i>C. bovis</i>	Cattle	EF493331	Brazil	Thomaz et al. (2008)
SSU-rDNA	Deer genotype	Deer	AY120910	USA	Xiao et al. (2002)
SSU-rDNA	<i>C. ryanae</i> (deer-like genotype)	Cattle	AY587166	USA	Santín et al. (2004)
SSU-rDNA	<i>C. ryanae</i>	Cattle	DQ871345	China	Feng et al. (2007)
SSU-rDNA	<i>C. ryanae</i>	Cattle	EU410344	USA	Fayer et al. (2008)
SSU-rDNA	<i>C. ryanae</i> (deer-like genotype)	Cattle	EU203216	USA	Feltus et al. (2008)
HSP-70	<i>C. bovis</i>	Cattle	AY741306	USA	Fayer et al. (2005)
HSP-70	<i>C. ryanae</i>	Cattle	EU410346	USA	Fayer et al. (2008)
HSP-70	<i>C. ryanae</i> (deer-like genotype)	Cattle	DQ182597	Denmark	Langkjaer et al. (2007)
HSP-70	Deer genotype	Deer	AY741308	USA	Fayer et al. (2005)
Actin	<i>C. ryanae</i>	Cattle	EU410345	USA	Fayer et al. (2008)
Actin	<i>C. ryanae</i> (deer-like genotype)	Cattle	AY741309	USA	Fayer et al. (2005)
Actin	Deer genotype	Deer	AY120928	USA	Xiao et al. (2002)
Actin	<i>C. bovis</i>	Cattle	AY741307	USA	Fayer et al. (2005)

for these three loci provide strong support that *C. xiaoi*, *C. bovis*, *C. ryanae*, and the deer genotype form a cluster but *C. xiaoi* is genetically distinct from the others (Figs. 1–3). Phylogenetic analyses of the SSU-rDNA locus revealed that *C. xiaoi* had a pairwise distance of 0.001–0.007 with *C. bovis*, 0.010–0.016 with *C. ryanae*, and 0.007–0.013 with the deer genotype. Recognized species of *Cryptosporidium* share similar differences such as *C. meleagridis* vs. *C. wrairi* (0.013), *C. parvum* vs. *C. hominis* (0.007), and *C. muris* vs. *C. andersoni* (0.007). Phylogenetic analyses of the HSP-70 locus revealed that *C. xiaoi* had a pairwise distance of 0.025 with *C. bovis*, 0.102 with *C. ryanae*, and 0.069 with the deer genotype. Recognized species of *Cryptosporidium* share similar differences such as *C. meleagridis* vs. *C. wrairi* (0.025), *C. parvum* vs. *C. hominis* (0.019), and *C. muris* vs. *C. andersoni* (0.022). Phylogenetic analyses of the actin locus revealed that *C. xiaoi* had a pairwise distance of 0.023 with *C. bovis*, 0.010–0.134 with *C. ryanae*, and 0.116 with the deer genotype. Recognized species of *Cryptosporidium* share similar differences such as *C. meleagridis* vs. *C. wrairi* (0.041), *C. parvum* vs. *C. hominis* (0.017), and *C. muris* vs. *C. andersoni* (0.030).

The SSU-rDNA gene of *Cryptosporidium* evolves slowly, with sequence variations limited to several regions of the gene. In contrast, the actin and the HSP gene are highly polymorphic over the entire length of the genes which explains the higher values obtained for these genes seen in the pairwise distance analyses between *Cryptosporidium* species. Because of the high sequence heterogeneity, the HSP-70 and the actin genes are probably more useful than the SSU-rDNA gene for inferring the genetic relationship of closely related *Cryptosporidium* species such as *C. bovis* and *C. xiaoi*. However, comparison at the HSP-70 and actin gene with other isolates was limited because of the lack of *C. xiaoi* (the *C. bovis*-like genotype or *C. bovis* isolated from sheep) sequences in GenBank.

Genetic differences from three independent loci combined with biological differences such as strict host specificity for sheep, and the lack of pathogenicity (in experimentally infected sheep) indicate that *C. xiaoi* is a distinct species. The correct identification of *Cryptosporidium* species in fecal specimens from farm animals has important veterinary and public health implications. Livestock of all ages and in all conditions of illness and well-being have been found infected with *Cryptosporidium*. Diagnosis of infection, usually by microscopy, unconfirmed by molecular methods, can lead to misinterpretation of sources of pathogenic zoonotic species such as *C. parvum* or the cervine genotype. Establishment of the species *C. xiaoi* from the former *C. bovis*-like genotype should help to clarify any confusion that exists between the names *C. bovis* and the *C. bovis*-like genotype.

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